SELECTIVE, SPECIFIC, AND VERSATILE PERSONAL BIOSENSORS TO ORGANOPHOSPHATE CHEMICAL TOXINS COMPOSED OF POLYURETHANE IMMOBILIZED ENZYMES

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ABSTRACT

Organophosphorus (OP) nerve agents are a serious threat to military and civilian personnel, so rapid detection of OP compounds in all of these forms is of paramount importance to prevent casualties. Recently, we combined porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and enzymes such as ChEs. One of the advantages of this technique imparted to the *immobilized* enzymes is resistance to denaturing events. Most important, the enzyme will not leach from the polyurethane support so the ChE-badge can now be used to sample for OPs in diverse environments such as soil and large bodies of water, as well as conventional sources such as air. In addition, immobilized enzyme badges are being designed with a unique attribute not present in the current non-immobilized detectors: a rapid field system to identifying which OP is present. For instance, organophosphorus hydrolase (OPH) hydrolyzes sarin more readily than soman, while laccase hydrolyzes the VX agent preferentially over the G agent OPs. Currently, we are evaluating polyurethane immobilized laccase for long-term stability and kinetic properties of VX hydrolysis. Thus, the immobilized sensor can provide new features and testing of more diverse environments than the M256A1 and M272 kits combined. Also, the ability to identify the OP toxin in real-time using the immobilized differential detector would aid in treatment and securing the contaminated area, in the identification of the use of OPs, and permitting first responders to identify the OP present in a civilian terrorist act.

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INTRODUCTION

Traditional analysis of cholinesterase inhibitors is performed using gas and liquid chromatography and mass spectrometry (Witkiewicz, et al., 1990). These techniques have significant drawbacks when considering an individual kit for field deployment, including lack of portability, simplicity, cost, and rapid results. An alternate technology is a biosensor. Biosensors have been widely used to de-

Covalent ChE Incorporation at Aliphatic Amino Group(s)

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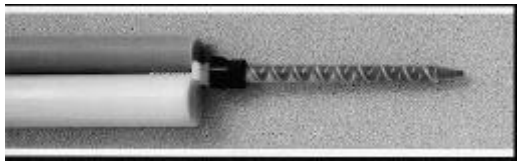


Figure 2. Apparatus for combining enzymes in aqueous buffer and prepolymer. Complete mixing is evident at the end of the mixing stator by the uniform and consistent gray (right).

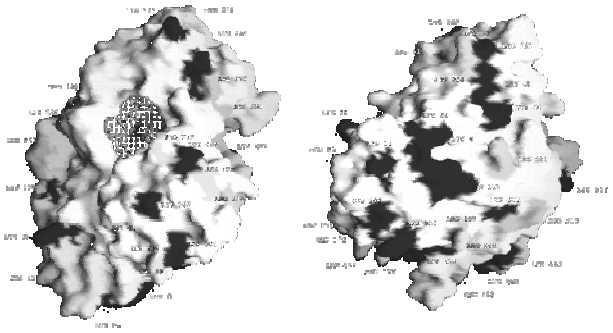


Figure 3. Computer model of the surface of butyrylcholinesterase based on the x-ray crystal structure. Left model: A speckled area in the middle represents the active site gorge through which substrate and OP must pass to get to the active site. Lysine residues on the surface of the enzyme (shown in black) are possible coupling sites for cross-linking to the prepolymer, and do not interfere with the gorge. Right model: 180^{o} rotation shows the backside of the enzyme and additional coupling sites to lysine moieties.

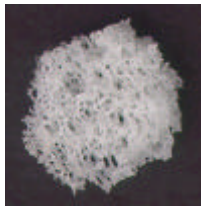


Figure 4. Biosensor, consisting of immobilized AChE and polyurethane polymer. The biosensor depicted here is about the size of a pencil eraser.

covalently have been prepared by a variety of processes (Ghindilis, et al, 1996). The drawback to these methods includes lack of enzyme stability at ambient conditions, leaching from the surface, sensitivity to denaturating conditions, and short half-life when in solution. Currently fielded spot chemical agent detector kits and even water test kits use dry eel ChE non-covalently applied onto fiber or ion-exchange paper. It can only be exposed to air/vapor or at most several drops of aqueous solutions. Wood and coworkers (Wood, et al., 1982), using isocyanate-based polyurethane prepolymers (Hypol®), found that a number of enzymes could be covalently bound to this polymer, and that every enzyme retained activity to varying degrees. We have combined a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and enzymes such as ChEs, producing immobilized enzyme sponges (Gordon, et al., 1999; Ember, 1997; Medlin, 1998). In this method, the enzyme becomes an integral part of the solid support (figure 1). Some of the advantages of this technique include retention of similar kinetic characteristics as the soluble form of the enzyme. Most important, the immobilized enzyme retains high activity after prolonged storage, and it is resistant to the detrimental effects of low and high temperatures, and long exposure to the environment. In addition, because the enzymes are covalently attached to the polyurethane, they will not leach from this polymer support so that the product - an OP badge - can now be used to sample for chemical weapons and pesticides in anything from soil, water, to air.

METHODS

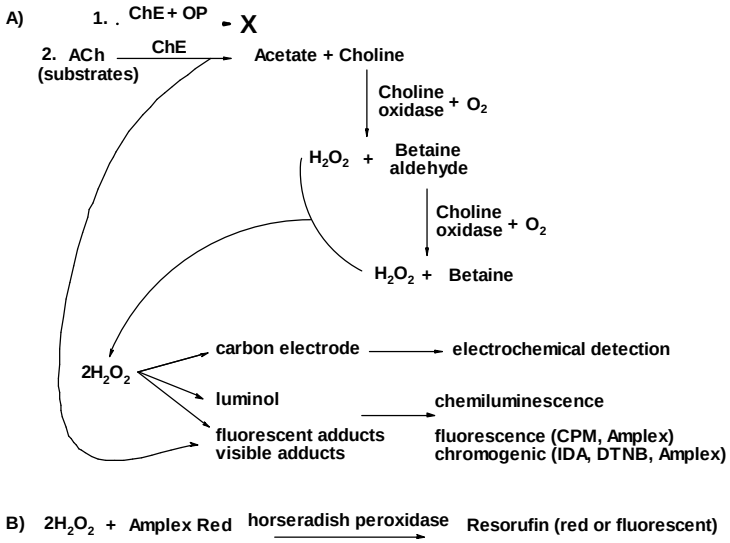


Figure 5. A) Schemes for detecting cholinesterase activity of the biosensor including visible, fluorescent, chemiluminescent, and electrochemical methods. In reaction 1, the biosensor ChE reacts with OP, and the enzyme is inhibited, so no change in color is generated. In reaction 2, uninhibited ChE cleaves the substrate, and can produce a variety of detection results. B) Specific mechanism for the biosensor to yield resorufin, both a visible red and a fluorescent indicator.

BASF, Specialty Chemicals, Parsippany, NJ) and an equivalent volume of water miscible prepolymer (TDI Hypol 3000 prepolymer, Dow Chemical, Lexington, MA). The 2-phase system, enzyme and prepolymer, is mixed by a method we modified from the adhesive industry. The mixing apparatus uses a 1:1 ratio double barrel chamber and a mixing stator (figure 2, CPA, Inc., 21 Starline Way, Cranston, RI 02921). The isocyanate functional group of the Hypol prepolymer reacts with the surface amine groups of enzymes (figure 1and 3; Gordon, et al., 1999; Ember, 1997).

Figure 3 depicts possible cross-linking sites between BChE surface lysine groups and the prepolymer. Similar computer models of AChE, OPH, and laccase demonstrate cross-linking sites that would not interfere with the active site of the enzymes. The mixed material ejected from the mixing stator can be ejected into a mold to form any size or shape product. Alternatively, the immobilized product can be spotted (as a dot of glue) onto a paper or rigid plastic backing, generating the immobilized ChE biosensor ticket. A 5 mg biosensor is shown in figure 4 (not to scale).

Determination of immobilized enzyme activity: Several different techniques to determine the activity of the immobilized ChE enzymes in the biosensor are shown in the scheme in figure 5. Detection can be performed qualitatively by the human eye for visible chromagens, or dark-adapted eyes for chemiluminescent chromagens (Parari, et al, 1993; Birman, 1985; Okabe, et al., 1977). Detection can also be performed quantitatively using portable handheld devices, which measure fluorescence, chemiluminescence, and visible chromagens. Typically, reactions of the immobilized enzymes were monitored spectrophotometrically, e.g., by the Ellman assay for ChEs (De La Hoz,, et al., 1986), in a cuvette containing a stir bar and the biosensor.

RESULTS

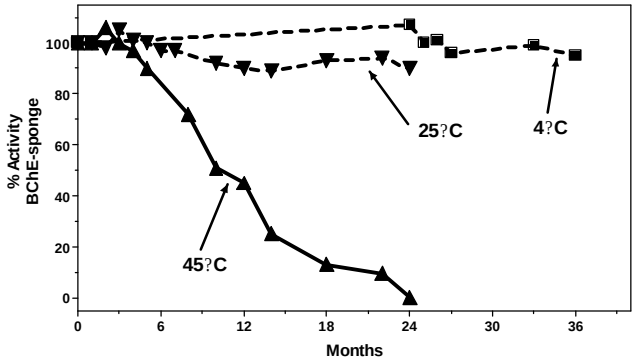


Figure 6. Long-term stability of BChE biosensor after continuous exposure to various temperatures.

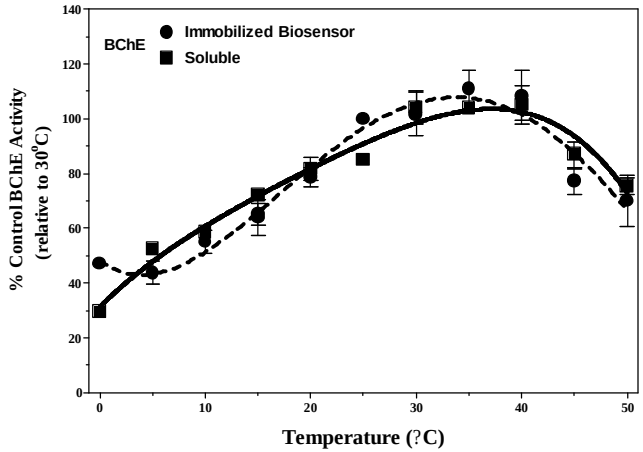
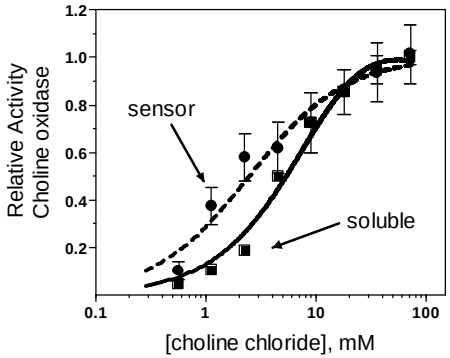


Figure 8. Relative reaction rates of soluble BChE and biosensor BChE at different temperatures relative to 30°C.



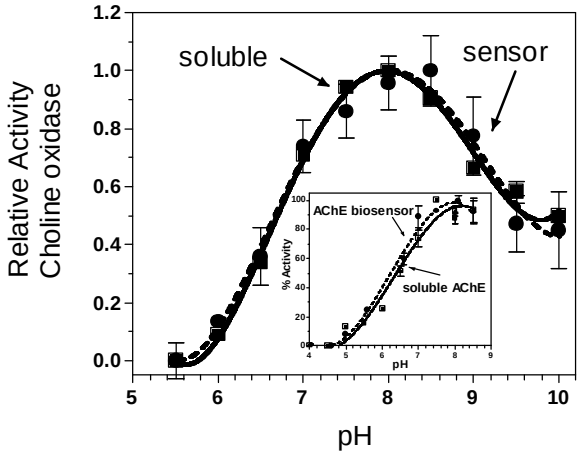
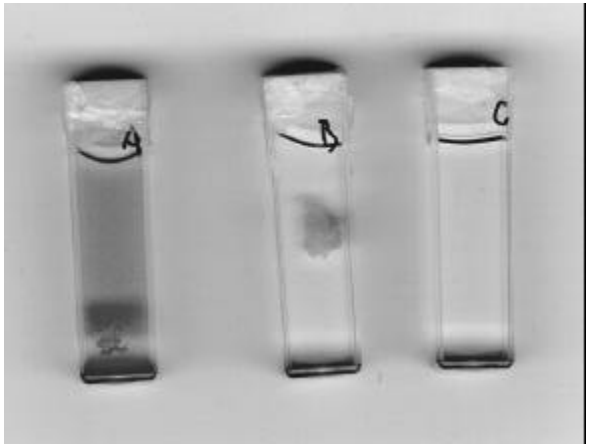


Figure 7. Top: Immobilized choline oxidase and soluble form of the enzyme display similar kinetic properties. Bottom: pH profiles of soluble and immobilized choline oxidase are identical and exhibit the same pH optimum as the AChE biosensor or soluble AChE.

Capacity of biosensor for multiple immobilized enzymes and coupled reactions: We found that the polymer badge has a significantly higher loading capacity for ChEs than the amount of purified BChE or AChE we added. Therefore, adding larger quantities of enzyme during synthesis could increase the final ChE activity of the biosensor. When increasing amounts of a nonspecific protein, such as bovine serum albumin that contains no ChE activity, were added to a constant amount of purified AChE and the mixture



AChE Sensor **OP - poisoned** **Blank**

Figure 9. Immobilized AChE biosensor after exposure to OP in aqueous solution using the resorufin indicator reaction. Tube A and B have AChE immobilized sensor. Tube B sensor was poisoned with OP prior to using the indicator; color developed was observed in tube A, but is lacking in tube B.

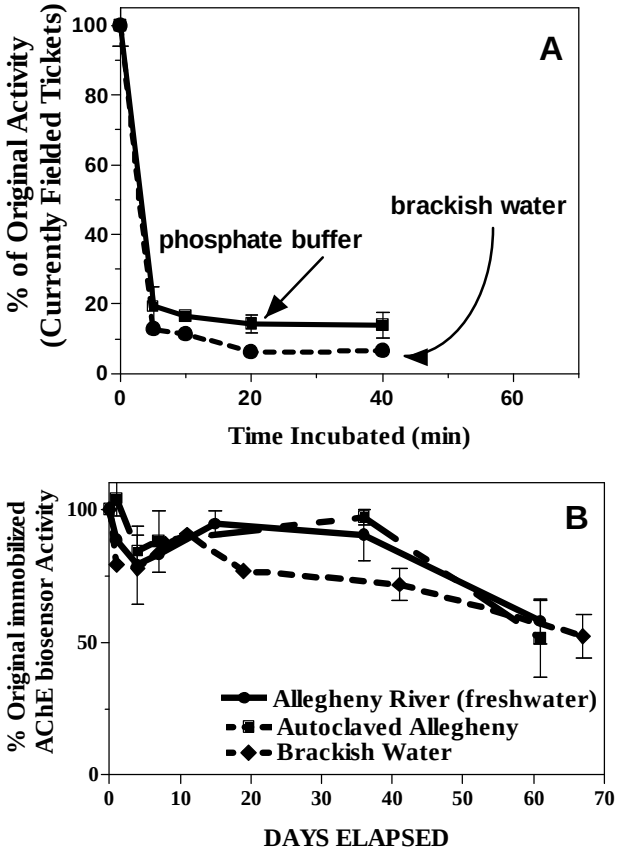


Figure 10. Comparison of a current *non-covalent* ChE ticket (A) and immobilized AChE biosensor (B) after exposure to aqueous solutions. Note that the time scale (x-axis) is minutes for the existing tickets and days for the immobilized AChE biosensor.

8), the coupled reactions depicted in the multiple assay scheme (figure 5) can be simultaneous optimized for both immobilized enzymes.

As expected for similar kinetic parameters, the relative rate of substrate hydrolysis and generation of color is identical for the soluble and immobilized ChEs, as shown in figure 8. While the enzyme is stable at significantly lower temperatures, both enzyme forms demonstrate temperature dependence.

Sensor color reactions: Figure 9 is an example of the biosensor poisoned by OP (tube B, middle), bio-

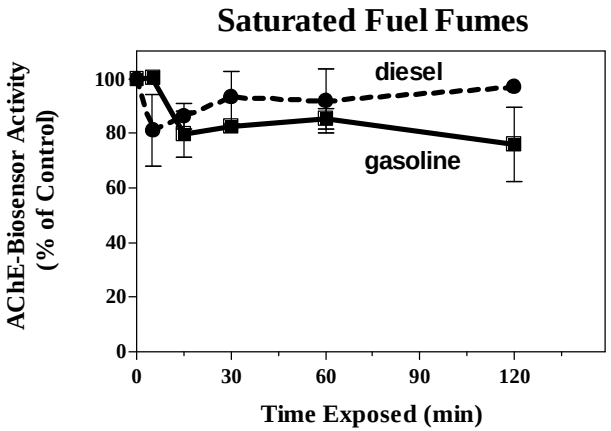


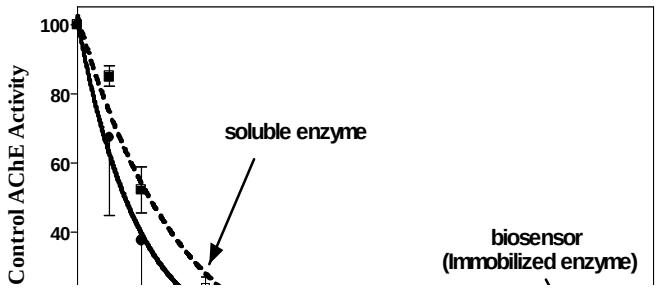
Figure 11. The biosensor (immobilized AChE) is resistant to false positives caused by continuous exposure to organic vapors, such as diesel and gasoline fumes.

TABLE 1. Time-Dependent Inhibition of ChEs by MEPQ

ChE	Enzyme Form	Bimolecular rate constant (M^{-1} min^{-1}) ± SD
AChE	soluble	$1.59 \pm 0.52 \times 10^{8}$
	biosensor	$1.00 \pm 0.28 \times 10^{8}$
BChE	soluble	$4.15 \pm 0.78 \times 10^{7}$
	biosensor	$4.21 \pm 2.00 \times 10^{7}$

do not dissociate (leach) from the polymer support. Therefore, the immobilized enzyme biosensor can be used to test water or even left in liquid or other environments for long-term monitoring.

Existing fielded OP tickets from the United States and other countries contain eel cholinesterase dried (not covalently attached) onto an ion-exchange filter paper. These tickets lost more than 80% of their original activity in less than *5 minutes* in various aqueous conditions, including pH 8 phosphate buffer or brackish water (figure 10A). Therefore, these tickets can only detect OPs in vapor or a *drop* of solution placed on the paper. In contrast to these tickets, the AChE activity in the immobilized biosensor was stable for almost 60 *days* in continuous immersion in aqueous samples including Allegheny River (fresh water, figure 10B) or brackish water. Since the results were identical for autoclaved and untreated river water, the immobilized enzymes were also resistant to microbial induced proteolytic degradation. Also note that the same biosensor was assayed multiple times over many days, so it is evident that the immobilization process confers dramatic stability to covalently coupled AChE, and provides further evidence that the enzymes do not leach from the polyurethane matrix.



The biosensor is less sensitive to events that cause false positives: In addition to robustness to temperature extremes and washout by aqueous environments, the biosensor is

Biosensor Sensitivity to Organophosphates: The biosensor composed of immobilized ChE and the soluble form of the enzyme exhibited the same titration curve to OP, as illustrated in figure 12. Furthermore, time-dependent inhibition of AChE and BChE by the organophosphate MEPQ yielded the same bimolecular rate constants of inhibition for soluble or immobilized AChE or BChE (Gordon, et al., 1999) (Table 1). These data demonstrate that the immobilized ChEs react to and detect OPs in the same manner as the soluble form of the ChEs, even though the biosensor has increased stability to adverse environmental assaults and is cross-linked to the polymer (figure 1).

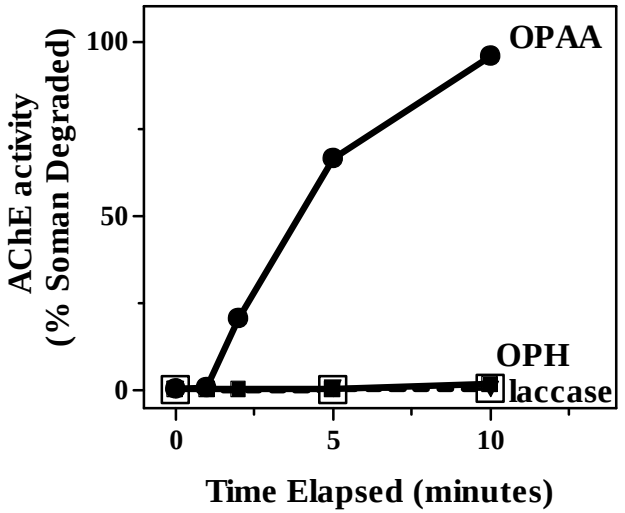


Figure 13. Differential hydrolysis of the OP soman. Soman is not rapidly hydrolyzed by OPH or laccase, which hydrolyze sarin and VX, respectively. In contrast, after 10 min OPAA hydrolyzed the soman completely so that now an AChE biosensor was no longer inhibited.

Differential OP Biosensor: The sensors we propose will have an additional unique attribute: We are developing a field system capable of differential identification of the type of OP contamination that occurred, e.g., sarin or soman. This would aid in the decontamination and treatment of OP contaminated individuals and permit tracking of OPs from a terrorist organization without removing samples to a central laboratory. The badge would be subdivided into compartments; each containing immobilized enzymes that behave uniquely to the different OPs (Gordon, et al., 1999). For instance, one strain of OPH hydrolyzes sarin more readily than soman, while OPAA hydrolyzes soman, and laccase hydrolyzes VX. To this end, we have successfully immobilized OPH, OPAA, and laccase using the same Hypol TDI prepolymer and conditions used for ChE immobilizations. In the first step, the solution containing a suspected OP (either in water, dirt, or after swabbing) would be added to a series of these differential immobilized enzymes. After 10 min, these enzymes would be removed as they are immobilized to the polyurethane, and the ChE-biosensor added. An uninhibited biosensor would indicate that an enzyme hydrolyzed the OP, and therefore which OP was present. This procedure can be seen in figure 13: The OP present was soman, based on time-dependent hydrolysis by OPAA, but not laccase or OPH.

CONCLUSIONS

We have demonstrated a unique immobilized enzyme biosensor system for the detection of OP contamination. This biosensor is versatile, durable, and reusable for sampling water and air or almost any environment. We have shown that the immobilized enzyme is active over 60 days, whereas existing OP

These immobilized enzyme badges have an additional unique attribute not present in the existing non-immobilized detectors: a rapid field system capable of identifying which OP is present. This is possible because the immobilization process stabilizes the enzymes that behave uniquely to different OPs. For instance, organophosphorus hydrolase (OPH) hydrolyzes sarin more readily than soman, while laccase hydrolyzes the OP VX preferentially over G-agent OPs.

The immobilized polyurethane enzymes will make versatile biosensors for detecting organophosphates. These badges, by virtue of their high capacity for most enzymes, stability, specificity, sensitivity, and resistance to harsh environmental conditions, can be used under diverse conditions encountered by troops in the field. These badges should be suitable for a variety of sensor schemes for both chemical weapons and pesticides. These badges should be suitable for first responders, Navy seals, and also civilian populations gathered in large numbers such as sports activities, subway stations, airports, crop dusters and farmers. They could be incorporated into the telemedicine initiative as electrochemical organophosphate probes when mated to an automatic reader.

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REFERENCES

1. Witkiewicz, Z., Mazurek, M., and Szulc, J. "Chromatographic analysis of chemical warfare agents." *J. Chromatogr*. Vol. 503 pp. 293-357, 1990.
2. Ghindilis, A. L., Morzunova, T. G., Barmin, A. V., and Kurochkin, I. N. "Potentiometric biosensors for cholinesterase inhibitor analysis based on mediatorless bioelectrocatalysis." *Biosensors Bioelectronics* Vol. ll pp. 873-880, 1996.
3. Wood, L. L., Hardegen, F. J., Hahn, P. A., Enzyme Bound Polyurethane. U.S. Patent 4,342,834, 1982.
4. Gordon, R K., Feaster .S R., Russell, A. J., LeJeune, K. E., Maxwell, D. M., Lenz, D. E., Ross, M., and Doctor, B. P. "Organophosphate skin decontamination using immobilized enzymes." *Chemico-Biological Inter*. Vol. 119-120, pp. 463-370, 1999.
5. Ember, L. "Detoxifying Nerve Agents", *Chemical & Engineering News*, pp. 26-29, (September 15, 1997).
6. Medlin, J. F. "Super Sponges." *Environ. Health Perspect*. 106:A182-A184, 1998.
7. De La Hoz, D., Doctor, B. P., Ralston, J. S., Rush, R. S., and Wolfe, A. D. "A simplified procedure for the purification of large quantities of fetal bovine serum acetylcholinesterase." *Life Sci*. Vol. 39, pp. 195-199, 1986.
8. Gan, H.K., Smolen, A., Eckerson, H.W., LaDu, A "Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities." *Drug. Metab. Disp*. Vol. 19, pp. 100-106, 1991.